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VERSITA

ACTA FACULTATIS PHARMACEUTICAE UNIVERSITATIS COMENIANAE

THE EVALUATION OF EFFICACY OF PYCNOGENOL® FRACTIONS ON ENDOTHELIAL DYSFUNCTION

HODNOTENIE ÚČINNOSTI FRAKCIÍ PYCNOGENOLU® NA ENDOTELOVÚ DYSFUNKCIU

Original research article

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Abstract The present study evaluates antihyperglycaemic activity of fractionated Pycnogenol® and its ability to improve endothelial dysfunction in diabetic animals. The aim of this study was to isolate from Pycnogenol® mixture its active compounds and compare their efficacy on observed parameters. Pycnogenol® mixture was fractioned by re-extracting with petroleum ether, chloroform, ethyl acetate and butanol, subsequently. Pycnogenol® mixture and fractions (butanolic, water, ethyl acetate) were administered from 6 weeks to diabetic rats. Blood glucose levels were assessed from the arterio-venous blood at the beginning of the experiment and at the end of the experiment. Endothelial dysfunction was evaluated as the contractile responses to phenylephrine and acetylcholine. The amount of collagen I and III was assessed from thoracic aorta after picrosirius red staining. For the confirmation of the changes on molecular level, we determined the endothelial NO synthase and heat shock protein 90 expression from left ventricle.
Overall, the result suggest that fractions are not so effective on observed parameters as Pycnogenol® mixture itself, indicating synergistic effect of the plant constituents.

Slovak abstract Táto štúdia bola zameraná na hodnotenie antihyperglykemickej activity frakcionovaného Pycnogenolu® a jeho schopností zlepšovať endotelovú dysfunkciu u diabetických zvierat. Cieľom štúdie bolo izolovať zo zmesi Pycnogenolu® aktívne zložky a porovnať ich účinnosť na vybrané parametre s účinnosťou celkovej zmesi. Celá zmes bola frakcionovaná vytrepávaním s petroléterom, chloroformom, etylacetátom a butanolom. Pycnogenol®, rovnako ako aj jeho frakcie (butanolová, vodná, etylacetátová), boli podávané počas 6 týždňov diabetickým zvieratám. Z artério-venóznej krvi boli stanovené hladiny glykémie pred začiatkom podávania látok a na konci experimentu. Endotelová dysfunkcia bola hodnotená ako reaktivita ciev na podaný fenylefrín a acetylcholín. Následne bolo stanovené množstvo kolagénu I a III z torakálnej aorty po farbení pikrosíriusovou červenou. Na potvrdenie zmien na molekulárnej úrovni sme stanovili expresiu endotelovej NO-syntázy a heat shock proteínu 90 v ľavej komore srdca. Dosiahnuté výsledky naznačujú, že frakcie nedosahujú účinnosť, ktorú preukázalo podávanie nefrakcionovaného Pycnogenolu®, za čo by mohol zodpovedať synergizmus jednotlivých zložiek zmesi.

Keywords *diabetes – Pycnogenol® mixture – fractions – oxidative stress – endothelial dysfunction*

Kľúčové slová: *diabetes – Pycnogenol® – oxidatívny stres – endotelová dysfunkcia*

1. Introduction

The pathogenesis of diabetes mellitus and its complication reflects the presence of oxidative stress produced as a result of hyperglycaemia (Jay et al., 2006). Hyperglycaemia associated with diabetes is usually controlled by diet management, oral hypoglycaemic agents and insulin therapy. It is a major cause for the development of diabetic complications such as endothelial dysfunction. These complications increase morbidity and mortality of patients; therefore, new approaches for their prevention or treatment are desirable.

For this reason, there is a huge interest for complementary and alternative medicine involving the use of traditional medicinal herbs and other dietary supplements (Ryan et al., 2001). One of the logical ways for searching new potentially efficient drugs is an evaluation of effects of plants and their active compounds (Choudhary et al., 2012).

Pycnogenol® (Pyc, Horphag Research Ltd, UK, Geneva, Switzerland) is a Pine bark extract from French maritime pine *Pinus pinaster* (*maritima*), which grows in the coastal southwest France. The quality of this extract is specified in the United States Pharmacopoeia. Its chemical composition is still not completely clear. The main constituents of Pyc are known to be phenolic compounds, broadly divided into monomers (catechin, epicatechin and taxifolin) and condensed flavonoids. Pyc has been demonstrated to have many effects

in experimental and also in clinical practice. Pyc is a strong natural antioxidant that is available in Slovakia as a supplement. There are studies that have proven its positive effects on diabetic microangiopathy (Cesarone et al., 2006), influence on the levels of superoxide dismutase (Kolacek et al., 2010) and tissues oxidative damages decrease by reducing oxidative stress levels, (Parveen et al., 2010). Besides lowering blood glycaemia and elevation of glutathione reductase levels it plays an important role in the protection of endothelium against oxidative stress induced by reactive oxygen and nitrogen species (Virgili et al., 1998). Endothelium-dependent vasodilatation is enhanced by NO. However, in diabetes this is response decreased because of elevated levels of free oxygen radicals. The changes in microcirculation represent most common pathologic findings in diabetic condition. The micro- and macro-vascular complications development belongs to the basic pathways leading to the organ damages and clinic symptoms in patients (Rosei & Rizzoni, 2010). One of the mechanisms causing decreased availability of NO to the vasculature is lowered expression and activity of endothelial NO-synthase (eNOS) (Wang & Marsden, 1995). Other mechanisms affected the availability of NO include the interactions of eNOS with its inhibitory protein caveolin-1 and activation with molecular chaperone heat shock protein 90 (Hsp90) (Chatterjeea & Catravas, 2008). Hsp 90 is known to regulate calcium-dependent dissociation of eNOS from caveolin-1. The binding of Hsp 90 to eNOS ensures the transition from the early Ca^{2+}-dependent to the late phosphorylation-dependent activation of eNOS. There is a study that has demonstrated that chronic exposure of endothelial cells to hyperglycaemia downregulate protein interaction between eNOS and Hsp90. The end result is the deactivation of eNOS and imbalance in NO versus reactive oxygen species levels (Lin et al., 2005).
Besides the changes in the expression of protein in endothelium, chronic oxidative stress is described to cause changes in the amount of collagen I and III in vascular wall. Typical finding is increased amount of collagen I and decreased amount of collagen III. Polyphenols, compounds that Pycnogenol® consists of, are known to lower the amount of collagen I and increase the levels of collagen III, thus preventing the changes caused by oxidative stress (Hlavackova et al., 2011).
Since Pycnogenol® exhibited adjuvant effect in combined therapy in several studies in different diagnosis (Stuard et al., 2010; Belcaro et al., 2010; Zibadi et al., 2008), we focused in this study on fractionating this mixture, because there are just a few studies in which Pycnogenol fractions were used. We were interested whether Pycnogenol fractions are more effective than whole mixture or not. We evaluated antihyperglycaemic activity and the impact of administration of Pycnogenol® fractions on endothelial dysfunction, changes of collagen amount and expression of proteins in diabetic animals.

2. Materials and methods

Animals

Male Wistar rats of 6-week old (200 g) were used in the experiment. They were of monitored conventional quality (Dobra Voda, Slovak Republik) and were housed in a quarantine facility for 8 days before use. During the whole experiment, the animals had free access to a standard commercial diet and water. The animal room was continuously monitored for the temperature of 23 ± 1°C and relative humidity of 40–70%. The animals were kept under a stable regimen of 12 hours light/12 hours darkness. Pycnogenol® was kindly donated by Dr. Minczinger (Generica s.r.o., Slovak Republic). The Pycnogenol® fractions were prepared by Doc. PharmDr. Mucaji, PhD. The animals were randomised into the groups (n= 4–8). The first group – control (CON) – consisted of healthy control rats. For induction of diabetes in the other groups, the *i.p.* injection of streptozotocin (STZ) (Sigma, St. Louis, MO, USA) was used over three sequential days in the dose of 25 mg/kg b.w. STZ was dissolved in 0.1 mol/l citrate buffer, pH 4.5. Animals in one diabetic group remained without treatment – untreated diabetic rats (D).

Treatment of animals

The treatment started 8 days after uncontrolled diabetes. During the sixth weeks, the animals were administered butanolic (DBF, 9.71 mg/kg b.w./day *p.o.*), water (DWF, 6.44 mg/kg b.w./day *p.o.*) or ethyl acetate (DEF, 3.76 mg/kg b.w./day *p.o.*) fraction of Pycnogenol®. One group was treated with whole mixture of Pycnogenol® (20 mg/kg b.w./day *p.o.*). To one group of healthy animals (CON) and one group of diabetic untreated animals (D) was given vehicle (distilled water).
The doses of fractions were counted according to the aliquot substitution in whole mixture and were obtained by extraction and following desiccation of whole mixture (Table 1). The basic dose of Pycnogenol we counted was 20 mg/kg/day, because it was the most effective dose based on our previous results (Jankyova et al. 2009).

Table 1. The substitution of fraction in the Pycnogenol® mixture

Diluent	Extract (g)	Extract (%)	Dose (mg)
Petroleum ether	0.0069	0.14	-
Chloroform	0.0155	0.31	-
Ethyl acetate	2.4273	18.81	3.76
Butanol	1.2050	48.55	9.71
Water	0.7989	32.19	6.44

All procedures involving the use of experimental animals were approved by the State Veterinary and Food Administration of the Slovak Republic. The investigation conforms to the Guide for the Care and Use of Laboratory Animals: Eight Edition (2010) published by the US Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council and to the EU adopted Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for experimental and other scientific purposes.

Determination of blood glucose

Blood glucose levels were determined during experiment by using a glucose kit (Sigma, St. Louis, MO, USA) and by spectrophotometric analysis. The blood samples were collected initially before first administration of drug and at the end of experiment before sacrificing the animals preprandially (after 12 hours starving) and postprandially by tail vein. Non-diabetic animals were excluded from further experiments after first measurement.

Determination of vascular contractility

After animals were sacrificed (5% thiopental 80 mg/kg b.w. *i.p.*), thoracic aorta was excised from the diaphragm to the arch as quickly as possible and placed into the isolated tissue bath containing Krebs-Henseleit solution. Adherent fat and connective tissue were removed. Three millimetre long segments cut from each segment using surgical blade were placed between two stainless steel hooks inserted into the lumen and placed into the aperture for isolated organs (TSZ-04 Multi Chamber Tissue Bath, Experimetria Ltd, Hungary) for isometric tension recordings.

The preparation was taken with the care to avoid the damage of the endothelium. The Krebs-Henseleit solution was heated at 37°C and was constantly aerated with pneumoxid (95% O_2, 5% CO_2). After equilibration (30 min., tension 1.5 g), the maximal contraction (100%) with KCl (80 mmol/l) based on depolarisation of membrane was achieved in 15 min.

After washout period, the endothelial function was tested by measurement of contraction responses provoked with phenylephrine (10^{-5} mol/l, Sigma-Aldrich, Saint Louis, MO, USA) and relaxation responses provoked with acetylcholine (Ach, 10^{-5} mol/l, Sigma-Aldrich, Saint Louis, MO, USA). The responses were transferred as a digital signal (FSG-01 Force/displacement transducer, Experimetria Ltd, Hungary) and recorded with the software S.P.E.L. Advanced ISOSYS (Experimetria Ltd, Hungary). Presented data are the average of the group, whereas two recordings from each animal were performed.

Determination of eNOS and Hsp90 expression

The expression of eNOS and Hsp90 was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. The tissue samples from left ventricles were quickly frozen in liquid nitrogen and homogenised according to Krenek et al. (2006). The protein concentrations were measured by the Lowry method and normalised to 50 µg per sample. Proteins were separated on SDS–PAGE and transferred to polyvinylidene fluoride transfer membrane (Immobilon-P, Millipore Corp., Billerica, MA, USA). After blocking with 5% nonfat milk, blots were probed with a mouse anti-eNOS and rabbit anti-Hsp90 (BD Transduction Laboratories, Franklin Lakes, NJ, USA). β-actin (Sigma-Aldrich, St Louis, MO, USA) was used as a marker to control for the variation in the quantity of the separated total proteins. The amount of the specific protein was detected by using chemiluminescent detection (ECL Plus, Amersham, Buckinghamshire, UK). Autoluminograms were scanned and processed densitometrically. The arbitrary density levels were normalised to the average control signal.

Histology of aorta

The abdominal aorta was precisely excised and fixed in formalin (10%, Chemko, a.s., Strazske, SR). After then it was processed in paraffin (Centralchem, s.r.o., Bratislava, SR) and cut into 5-µm thick slices. They were stained with haematoxylin. For the assessment of the amount of collagen I and III, the slices were stained with a modified technique with picrosirius red as described previously (Hlavackova et al., 2011). Five places were randomly selected on each slide and viewed under polarised light. They were documented with a digital photographic camera S50 (Canon, Japan) and evaluated with ImageJ software (National Institute of Health, Bethesda, USA). Threshold values were determined for the particular colours of spectrum (0–35 for red, corresponding to collagen I and 45–110 for green colour, corresponding to collagen III). The numbers of pixels of each colour were counted and the percentage of the picture's area was calculated.

3. Results

Effect of Pycnogenol® and its fraction on body weight and on glycaemia

A weight loss was found in a diabetic untreated animals compared with healthy control rats. Neither the administration of Pycnogenol® mixture nor its fraction could prevent this loss (Table 2).

Diabetic untreated animals had significant increase in blood glucose levels compared with healthy control group.

The administration of Pycnogenol® mixture and butanolic fraction significantly lowered hyperglycaemia. Water fraction showed minor effect on hyperglycaemia compared with the effect of Pycnogenol® mixture. Ethyl acetate fraction administration was inefficient on lowering blood glucose levels (Table 2).

Effect of Pycnogenol® and its fraction on vascular contractility

The endothelial function was assessed as the measurement of the contractile responses of the thoracic aorta *in vitro* (Fig. 1). The maximal contraction (100%) was found to be the contraction after KCl. The contraction responses of untreated diabetic rats were increased compared with healthy control (CON:

Table 2. Body weight and glycaemia in experimental animals

	Body weight (g)		Glycaemia (mmol/l)	
Group	Initial	Final	Preprandial	Postprandial
CON	208.75 ± 7.47	385.00 ± 13.99	4.68 ± 0.40	8.24 ± 0.22
D	210.00 ± 7.36	352.50 ± 12.99	8.44 ± 0.37^{+++}	28.65 ± 2.65^{++}
DBF	200.00 ± 6,95	360.00 ± 17.84	7.58 ± 0.67	14.96 ± 4.32†
DWF	197.50 ± 5.44	360.00 ± 8.85	8.23 ± 1.00	19.89 ± 4.32
DEF	194.17 ± 5.39	364.17 ± 10.91	11.95 ± 3.02	28.52 ± 4.00
DP	205.83 ± 7.46	344.17 ± 15.19	7.12 ± 0.84	15.23 ± 3.54†

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs.* initial.
$^{++}p < 0.01$; $^{+++}p < 0.001$ *vs.* CON.
$^{\dagger}p < 0.01$ *vs.* D.

80.22 ± 5.65% *vs.* D: 99.22 ± 2.48%, $p < 0.05$). Untreated diabetic rats had impaired relaxation responses after acetylcholine treatment compared with healthy control group (CON: 67.78 ± 11.78% *vs.* D: 61.37 ± 1.61%, $p < 0.01$).

The administration of Pycnogenol® mixture, butanolic and ethyl acetate fraction showed decrease in vascular contraction (DBF: 56.93 ± 3.79%, $p < 0.001$, DEF: 68.35 ± 7.37%, $p < 0.05$, DP: 66.82 ± 7.42%, $p < 0.05$ *vs.* D). Treatment of diabetic rats with Pycnogenol® mixture and all fractions showed positive effects on the relaxation of vessels (DBF: 91.49 ± 8.04%, DWF: 78.62 ± 7.65%, DEF: 85.61 ± 9.08%, DP: 96.29 ± 12.97%, $p < 0.05$ *vs.* D).

Effect of Pycnogenol® and its fraction on the level of collagen

Both diabetic untreated and treated animals showed decrease in the amount of collagen I in the aortic wall compared with healthy control animals (CON: 7.44 ± 0.91%, D: 6.45 ± 0.75%, DBF: 5.80 ± 0.53%; DWF: 6.07 ± 0.59%; DEF: 4.28 ± 0.32%, Fig. 2). However, this decrement was not statistically significant. The level of collagen III was significantly decreased in diabetic untreated animals in comparison to healthy control animals (CON: 1.94 ± 0.27% *vs.* D: 0.97 ± 0.15%; $p < 0.05$). Pycnogenol® treated group has significantly increased level of collagen III compared with untreated diabetic animals (DP: 2.11 ± 0.40%, $p < 0.01$). The treatment with the Pycnogenol® fractions showed the trend to increase in collagen III although without statistical significance (DBF: 1.54 ± 0.14%, DWF: 1.38 ± 0.10%, DEF: 1.31 ± 0.15%). The ratio of collagen I/III was increased in untreated diabetic animals compared with healthy control group (CON: 4.18 ± 0.38 *vs.* D: 7.75 ± 0.88; $p < 0.001$). This increase indicates elevated rigidity of aorta, because collagen I is firmer than collagen III. The administration of Pycnogenol® itself and its fraction led to significant decrease of the collagen I/III ratio (DBF: 4.15 ± 0.31, $p < 0.001$; DWF: 4.41 ± 0.25, $p < 0.001$; DEF: 4.05 ± 0.30, $p < 0.001$; DP: 4.01 ± 0.40, $p < 0.001$ *vs.* D).

Effect of Pycnogenol® and its fraction on the eNOS and Hsp90 expression

The levels of eNOS (Fig. 3) were decreased in diabetic untreated animals compared with control group (CON: 100.00 ± 17.14%, D: 49.85 ± 5.17%). The administration of Pycnogenol® fraction did not show any positive effect on expression of eNOS (DBF: 37.22 ± 5.60%, DWF: 72.09 ± 12.28%, DEF: 42.45 ± 6.04%). The only positive significant effect was achieved with administration of Pycnogenol® mixture. The expression of eNOS was increased significantly compared with diabetic untreated animals (DP: 159.31 ± 18.45%, $p < 0.05$ *vs.* D).

The assessment of Hsp90 (Fig. 3) demonstrated increased expression in untreated diabetic rats compared with healthy control animals (CON: 100.00 ± 9.43% *vs.* D: 148.44 ± 12.58%, $p < 0.05$). The administration of ethyl acetate fraction and Pycnogenol® mixture (DEF: 104.58 ± 8.91%, DP: 107.20 ± 9.91%, $p < 0.05$ *vs.* D) was found effective in lowering the Hsp90 levels.

4. Discussion

Various studies were performed for testing Pycnogenol® effects in the treatment of diabetes and its complication. Our previous studies (Jankyova et al., 2009, 2012; Klimas et al., 2010) showed positive effects of Pycnogenol® mixture on different parameters of diabetic damages (e.g. diabetic neuropathy, diabetic cardiomyopathy, expression of iNOS, gp91^{phox}). We focused this study on uncovering the most effective fraction of the Pycnogenol® mixture that could have the higher selectivity of the active substances and thus have a greater influence for prevention and treatment of diabetic complications, especially endothelial dysfunction. Standardized Pycnogenol® mixture was separated according to the solubility in different solvents. There was no evidence about such separation of Pyc; however, Cheynier et al. (1998) used size separation of Pycnogenol® mixture by normal phase high-performance liquid chromatography.

The rats were administrated the three main fraction of Pyc – butanolic, water and ethyl acetate to diabetic animals for 6 weeks perorally and evaluated their effects on blood glucose levels, contractile responses of isolated aortic rings, the amount of collagen in aorta and the expression of proteins eNOS and Hsp90. The results were compared with the effects caused by Pycnogenol® mixture.

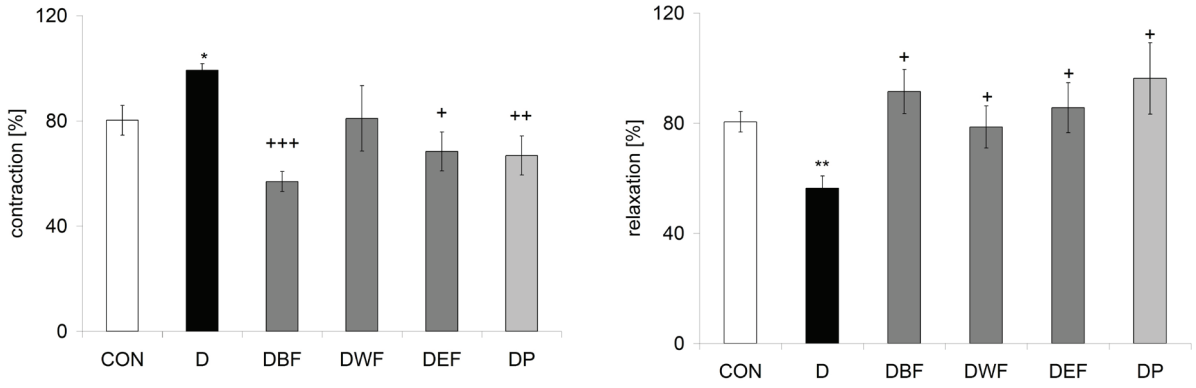

*Figure 1. Contractile responses of isolated thoracic aorta. CON – healthy control animals, D – untreated diabetic animals, DBF – diabetic animals treated with butanolic fraction of Pyc, DWF – diabetic animals treated with water fraction of Pyc, DEF – diabetic animals treated with ethyl acetate fraction of Pyc, DP – diabetic animals treated with Pycnogenol® mixture. Values are expressed as mean ± standard error of the mean (n = 4–8). *p < 0.05 vs. CON. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. D.*

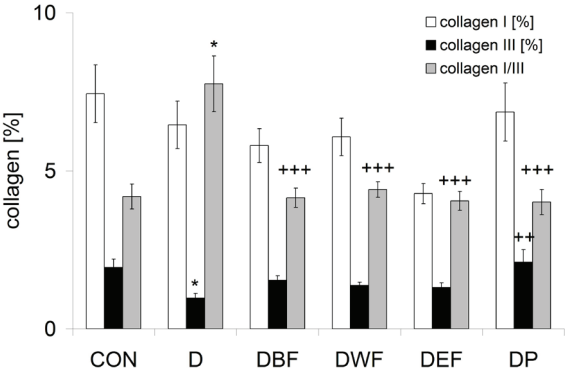

*Figure 2. The amount of collagen I, III and the ratio collagen I/III. CON – healthy control animals, D – untreated diabetic animals, DBF – diabetic animals treated with butanolic fraction of Pyc, DWF – diabetic animals treated with water fraction of Pyc, DEF – diabetic animals treated with ethyl acetate fraction of Pyc, DP – diabetic animals treated with Pycnogenol® mixture. Values are expressed as mean ± standard error of the mean (n = 4–8). *p < 0.05 vs. CON, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. D.*

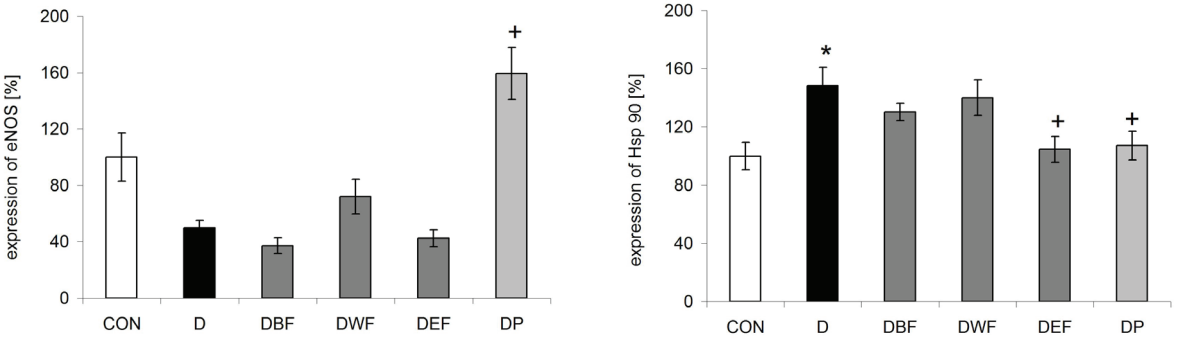

*Figure 3. The expression of eNOS and Hsp90 in experimental animals. CON – healthy control animals, D – untreated diabetic animals, DBF – diabetic animals treated with butanolic fraction of Pyc, DWF – diabetic animals treated with water fraction of Pyc, DEF – diabetic animals treated with ethyl acetate fraction of Pyc, DP – diabetic animals treated with Pycnogenol® mixture. Values are expressed as mean ± standard error of the mean (n = 3–4). *p < 0.05 vs. CON. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. D.*

In this experiment, we have proved the ability of butanolic fraction to lower the postprandial glycaemia to the level compared with healthy control animals as well as the levels of glycaemia attained with the treatment with Pycnogenol® mixture. Other fractions did not show any effects on lowering blood glucose levels. Pycnogenol® was described to lower the glycaemia in human as well (Liu et al., 2004). The mechanism of Pycnogenol® action on hyperglycaemia remains still unclear. There is possible involvement of mechanisms connected to its strong antioxidant properties such as increasing of antioxidant enzymes expression or the inactivation of circulating reactive oxygen species, similarly to another antioxidants (L-ascorbic acid, α-tocopherol) (Hamden et al., 2009). Another possible mechanism could be related to an inhibition of α-glucosidase (Schäfer & Högger, 2007). There is also the possibility of the direct effect of Pyc on pancreas, where it could increase insulin release analogous to equivalent plants (Kasiviswanath et al., 2005; Maiti et al., 2005) and synthetic derivatives of flavonoids, respectively (Bozdag-Dundar et al., 2001). Another plausible mechanism of decreased blood glucose levels could be Pyc's ability to increase the activity of hexokinase as a result of its protein binding (Packer et al., 1999) as in some other plants (Sekar et al., 2004).

In diabetes, there is decreased dilatation due to diminished bioavailability of NO resulting from increased production of reactive oxygen species (Cai & Harrison, 2000). Besides impaired dilatation, enhanced vasoconstriction after vasoconstrictive agents occurs in diabetes (Chang & Stevens, 1992).

In our study, contraction responses were increased in untreated diabetic animals. The administration of fractions and Pycnogenol® mixture caused improvement of contraction responses towards healthy control animals.

On the other side, untreated diabetic animals showed decreased relaxation of isolated aorta after acetylcholine compared with healthy control rats. The impaired relaxation response in diabetic rats could be to clarify with incorporation of advanced glycation end-products (AGEs) into subendothelial collagen that neutralizes NO released from endothelium before it reaches the smooth muscle of vessels and causes relaxation (Bucala et al., 1991). We confirmed the changes in the collagen amount as increased ratio of collagen I/III in vessels of untreated diabetic animals leading to elevation of vascular rigidity. Both Pycnogenol® mixture and its fractions decreased elevated ratio of collagen I/III to the level of healthy control animals. However, this depression was not followed by absolute improvement of relaxations. This reflects the presence of other pathophysiologic pathways in the progression of endothelial dysfunction. We testified the assumption that antioxidants could improve endothelium-dependent vasodilatation as the result of oxidative stress levels adjustment. The administration of Pycnogenol® mixture led to improvement of vasodilatation. Similarly, ethyl acetate fraction administration increased the relaxation responses in aorta. Fitzpatrick et al. (1998) have found that Pyc induces endothelium-dependent vasodilatation *in vitro* via mechanism of increase NO levels by enhancing the production of NO synthase. Still remains unclear, whether it is directly the ability to elevate NO-synthase synthesis, or it is just a result of NO-synthase protection caused by antioxidative effects of phenolic compound of Pyc (Fitzpatrick et al., 1998).

The expression of eNOS was decreased in untreated diabetic animals in our study. The insignificant result was caused probably due to small number of rats in control group; thus, there would be a need to repeat the assessment of eNOS expression with larger group of animals to confirm statistical significance. However, our results suggest that neither one fraction could effectively increase the eNOS expression. This corresponds with Hsp90 findings, in which just ethyl acetate fraction decreased the levels of Hsp90. Both eNOS and Hsp90 expressions were improved by administration of Pycnogenol® mixture. The results of various studies oriented on expression and activity of eNOS in animal models or in human are contradictory. There are studies that declare either decreased eNOS expression (Srinivasan et al., 2004; Davis et al. 2006) or increased eNOS expression (Cosentino & Lüscher, 1997). In diabetic animals, there is also occurrence of increased expression of mRNA, increased activity of proteins (Pieper et al., 1997) and decreased cGMP production (Lin et al., 2002). Failure of the eNOS–Hsp90 binding has been demonstrated to cause eNOS uncoupling and increased eNOS-dependent superoxide anion production.

This elevation causes production of peroxinitrate via reaction between NO and superoxide. As a result, the eNOS activity decreases and endothelial damage develops (Elrod et al., 2006). It is possible that Pyc as a strong antioxidant diminishes the production of superoxide and thus the expression of uncoupled eNOS (Maritim. et al., 2003). The enhanced association between eNOS and Hsp90 through Pyc could cause increased activity in endothelial cells of aorta that result in improvement of endothelial dysfunction, in our study, improvement of vasodilatation.

5. Conclusion

All the data taking together lead to the conclusion that Pycnogenol® had higher activity as mixture than the individual fractions. Our assumption that some of the fractions would be more efficient did not confirm, although they showed some positive effects on observed parameters, indicating a synergistic effect of the plant constituents.

6. Acknowledgement

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